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Microinjection of an Antibody to the Ku Protein Arrests Development in Sea Urchin Embryos

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Abstract. Ku is the regulatory subunit of the DNA-dependent protein kinase (DNA-PK). This enzyme plays a role in DNA repair, recombination, and transcription. It is composed of a large catalytic subunit (p460), and a regulatory heterodimer, the Ku protein, which consists of 86-kDa and 70-kDa subunits. These various components of the enzyme have been found in both eggs and embryos of the sea urchin. When variable amounts of a specific monoclonal antibody to the Ku protein (Ku 162) were injected into one cell of a 2-cell embryo of *Lytechinus pictus*, they caused a dose-dependent developmental arrest of the injected cell. The non-injected cell continued to develop normally. In contrast, injection of an antibody (N3H10) raised against the 70-kDa subunit of the Ku protein had no effect on development when injected into 2-cell-stage embryos. Co-injection of purified DNA-PK with the antibody reversed the antibody-mediated inhibition of development. In the fertilized egg and during the early stages of development, the DNA-PK was localized largely in the cytoplasm, but in later developmental stages, it assumed a nuclear location. On the basis of these results, we postulate that the injection of the Ku antibody either prevents the translocation of the DNA-PK into the nucleus or interferes with its enzymatic activity either in the nucleus or in the cytoplasm. In either case, the results suggest that DNA-PK plays an important role in regulating the early stages of embryogenesis in this primitive organism.

Introduction

The DNA-dependent protein kinase, DNA-PK, was first detected in the nuclei of mammalian cells (Walker *et al.*, 1985; Carter *et al.*, 1990). Studies in the last several years have provided evidence that this enzyme is involved in DNA repair, and in the activation of specific transcription factors (reviewed by Anderson and Lees-Miller, 1992; Weaver, 1995). It may also be involved in regulating DNA synthesis (Brush *et al.*, 1994), Ku-associated ATP-dependent helicase activity (Tuteja *et al.*, 1994), and DNA-dependent ATPase activity (Vishwanatha and Baril, 1990; Cao *et al.*, 1994). The enzyme consists of a large catalytic subunit, p460, and a heterodimeric regulatory component, the Ku protein. The latter consists of subunits of 86 and 70 kDa (Anderson, 1993; Gottlieb and Jackson, 1993). The Ku protein possesses a DNA-binding domain, and enzymatic activity is thought to be induced when this domain binds double-stranded DNA, (ds)DNA (Anderson and Lees-Miller, 1992; Dvir *et al.*, 1992; Anderson, 1993; Gottlieb and Jackson, 1993). However, the catalytic subunit of DNA-PK can be activated by another DNA-binding protein, heat shock transcription factor 1 (Peterson *et al.*, 1995). Additional complexity of control may be involved in its catalytic function, because the p86 subunit of Ku is a somatostatin receptor protein that can regulate the activity of protein phosphatase 2A (Le Romancer *et al.*, 1994). In addition, it has recently been reported that in cultured mammalian cells, a significant amount of the Ku protein is present in the cytoplasmic portion of the cell (Fewell and Kuff, 1996). These findings have led to the proposal that one or both of the Ku subunits may function in ways other than through the activation of nuclear DNA-PK, or that DNA-PK can be activated by factors other than (ds)DNA and function to regulate events in the cytoplasmic domain.

More recent work has shown that the enzyme is present in lower organisms, and particularly in the oocytes, eggs, and embryos of several marine invertebrates (Walker *et al.*, 1985; Kanungo *et al.*, 1996a, b) as well as in mice, frogs, and

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Drosophila (Finnie *et al.*, 1995). Thus, DNA-PK may have one or more evolutionarily conserved functions. Transgenic mice that are deficient in the Ku 86 subunit exhibit severe combined immunodeficiency (Nussenzweig *et al.*, 1996; Zhu *et al.*, 1996). Such mice are considerably smaller (40%–80%) than their normal littermates, and cultured cells derived from these mice display variable degrees of delay in cell cycle progression (Nussenzweig *et al.*, 1996).

That high concentrations of DNA-PK exist in the eggs of sea urchins (Kanungo *et al.*, 1996a, b) and frogs (Kanungo *et al.*, 1997) raises the interesting possibility that this enzyme may also play a regulatory role in early embryogenesis in these species. To explore this possibility, we initially undertook studies of DNA-PK localization and activation before and after fertilization of the eggs of *Arbacia punctulata*, the purple sea urchin. The unfertilized egg of this organism contains considerable amounts of both Ku and p460, as measured by western analysis, and by immunocytochemistry (Kanungo *et al.*, 1996b). These enzyme subunits are located largely, if not entirely, in the cytoplasmic compartment of the egg. Nonetheless, in the unfertilized egg, the DNA-PK cannot be activated by the addition of (ds)DNA to cytoplasmic extracts; *i.e.*, the enzyme is present in the cytoplasm in some cryptic form (Kanungo *et al.*, 1996b). Within minutes of fertilization, and without evidence of new protein synthesis, the enzyme is still in the cytoplasmic fraction, but can now be activated when (ds)DNA is added to this fraction. Of equal interest is that, if one coats Protein A Sepharose (PAS) beads with a specific monoclonal antibody to the Ku protein, Ku 162, and then uses these beads to isolate the Ku proteins from the cytoplasmic extracts of fertilized eggs, the beads bind the holoenzyme, and in this bound form the enzyme is active in the absence of (ds)DNA.

As the fertilized egg develops, there is a progressive shift of the various protein components of DNA-PK from a cytoplasmic to a nuclear location within the cell (Kanungo *et al.*, 1996b). The enzyme is located exclusively in the nuclear domain from the blastula stage onward, but in embryos at the 2- and 4-cell stages the enzymatic activity remains largely in the cytoplasm (Kanungo *et al.*, 1996a, b). In this report, we demonstrate that microinjection of the antibody, Ku 162, into one cell of a 2-cell embryo of *Lytechinus pictus*, the white sea urchin, inhibits the further development of the injected cell, but has no effect on the non-injected cell. This species, rather than *Arbacia punctulata*, was employed in the present experiments because the microinjection experiments were more easily performed in its embryos.

Materials and Methods

Collection of eggs and embryos. Male and female *Lytechinus pictus* were obtained from the Marine Resources Department of the Marine Biological Laboratory, Woods Hole, Massachusetts. Shedding of eggs and sperm was induced by injecting 0.5 ml of 0.5 *M* potassium chloride into the coelom. Batches of eggs were inseminated by mixing them with diluted sperm. Embryos were collected at scheduled times.

Kinase assay. Cytoplasmic extracts from the 2-cell embryos of *L. pictus* were prepared following the procedures already described (Ballinger *et al.*, 1984; Kanungo *et al.*, 1996a). Twenty-five units of purified human DNA-PK (Promega) or extracts prepared from *L. pictus* 2-cell embryos (from 250 embryos) was added to the antibody-coated PAS beads (Pharmacia) in a 500-μl volume made up by kinase assay buffer, and incubation was carried out at 4°C with constant mixing for 2 h. The PAS beads were washed six times with kinase assay buffer. The assay consisted of 12 μl of kinase assay buffer containing DTT (dithiothreitol) at a final concentration of 1 m*M*, 200 μM of peptide substrate (EPPLSQEAFADLWKK) (Anderson, 1993), 2 m*M* of $MgCl_2$, 130 m*M* of ATP, and 10 μCi of gamma-[32P]ATP (3000 Ci/mmol) (NEN, Du Pont). The assay was carried out at 25°C for 30 min and stopped by adding glacial acetic acid to a final concentration of 30%. The reaction product was spotted onto p81 phosphocellulose discs (Whatman). Several washes with 15% acetic acid following a 30-min wash with 30% acetic acid were carried out. The discs were finally washed for 5 min in acetone, air-dried, and the uptake of radioactivity was assessed by scintillation counting. The counts obtained from the control beads, not coated with any IgG but treated with either the purified human DNA-PK or extracts of *L. pictus* embryos, were used as a means of measuring nonspecific background radioactivity. These values were subtracted from the counts obtained from reactions using mouse IgG- and Ku 162-coated PAS beads.

Immunoprecipitation. DNA-PK holoenzyme was immunoprecipitated with PAS beads that had been coated with the Ku antibody as follows: PAS beads were pre-swollen in kinase assay buffer (50 m*M* HEPES, pH 7.4; 10 m*M* EGTA, 40 m*M* NaCl, 100 m*M* potassium acetate, 8.5 m*M* $CaCl_2$, 2.29 m*M* $MgCl_2$, 277 m*M* glycerol). Two micrograms of preimmune mouse IgG or of a monoclonal antibody to human Ku (Ku 162) that recognizes a conformational epitope of the Ku protein (Neomarkers, CA) was added to a 10-μl packed volume of PAS beads. After 1 2-h incubation at 4°C with constant mixing, the beads were washed four times with kinase assay buffer.

Immunoblotting. The immunoprecipitates from PAS beads were eluted by boiling in SDS-PAGE sample buffer and resolved on a 7.5% SDS-PAGE column (Laemmli, 1974). Duplicate gels were run. One was employed to prepare autoradiographs. The proteins from the other gel were transferred to nitrocellulose membrane and immunoblotted (Towbin *et al.*, 1979) with anti-p460 antibody as previously described (Kanungo *et al.*, 1996b).

Preparation of antibodies for microinjection. The IgG antibodies (Ku 162 and N3H10) and the control IgGs were used for the microinjection experiments (Wang *et al.*, 1993). They were a generous gifts from Dr. Westley Reeves of the University of North Carolina, Chapel Hill. Antibodies against RNAP II were obtained from Promega, and preimmune mouse IgG was purified using a Pierce immunoglobulin purification kit (Cat # 44667). The purified IgGs were dialyzed against Ca^{2+}-, Mg^{2+}-free PBS (Silver, 1986) and concentrated to 3 μg/μl. The antibody-enzyme complex was prepared using 6 μg of Ku 162 antibody and 10 μg of purified human DNA-PK (Promega) and incubated on ice with intermittent mixing for 2 h. The mixed aliquot was diluted to 1 ml with Ca^{2+}-, Mg^{2+}-free PBS, then dialyzed (4 × 2 liters) overnight. The dialyzed antibody-enzyme complex was collected and concentrated using an Amicon filter concentration unit. The final concentration was brought back to the original volume of the antibody solution used (10 μl), giving a final concentration of 600 ng/μl Ku 162 and 1000 ng/μl DNA-PK.

Microinjection. Specimens of *L. pictus* were obtained from Marinus Inc. (Long Beach, CA). To induce a female to shed eggs, about 0.5 ml of 0.5 *M* KCl was injected into the intracoelomic cavity. The eggs were then passed through an 80-μM diameter Nitex membrane to remove the jelly that surrounded them and washed once in Ca^{2+}-containing seawater. A 0.5-ml sample of eggs was placed onto a glass coverslip previously coated with poly-L-lysine, and the eggs were exposed to sperm. Low densities of activated sperm (1/40,000 dilution of dry collected sperm) were used to prevent the occurrence of polyspermy. If fertilization proceeded with an efficiency of at least 90%, we continued the experiment. After fertilization, the dishes were covered and left at 17°–19°C for 1 h, at which time the embryos begin first division. Solutions of antibodies that had been dialyzed and concentrated to known protein concentrations (3 μg/μl in the pipette, estimated as a final amount of 15×10^{-12} g in the cell) were microinjected into one cell of the 2-cell-stage embryo shortly after first cleavage and before aster formation of the second cleavage. We typically injected 1%–2% of the volume of the embryo. An occasional embryo was damaged by the microinjection procedure; such clearly damaged embryos were discarded. The injected embryos were again covered and remained on the microscope stage for a further 2 h; we then determined the incidence of continued or arrested division. In most experiments, digital images of the eggs were recorded using either an integrating cooled CCD camera (Hamamatsu, USA) or an intensified CCD camera (Photonics Science, Robertsbridge, UK). The camera was attached either to a Metamorph (Universal Imaging, USA) system controlled by a Pentium PC or an Ionvision (Improvision, Coventry, UK) system run by a Macintosh. In all experiments we used the uninjected cell as an internal control.

Immunocytochemistry. Embryos at the 2-cell stage immediately after antibody injection were fixed in 3% formaldehyde (Kanungo *et al.*, 1996b) and processed for indirect immunofluorescence by incubating with fluorescein conjugated anti-mouse IgG (Sigma Cat # F 0257). Embryos were then whole-mounted onto glass coverslips and viewed with epifluorescence and appropriate filters using a Zeiss 135 inverted microscope (Zeiss, Oberkochen, Germany). Pictures were recorded using a cooled CCD camera and collected digitally with Metamorph (Universal Imaging, West Chester, PA). The DNA of some of the injected cells arrested as a consequence of Ku 162 administration were counter-stained with a DNA stain, Hoechst 3342 (0.001 mg/ml, Molecular Probes, Oregon) to determine the structure of the DNA, and specifically to answer the question of whether the failure of these cells to undergo cell division could be due to the induction of apoptosis as a result of the antibody.

Results and Discussion

To determine whether DNA-PK plays a unique role during early embryogenesis, we microinjected two anti-Ku antibodies, both raised against human Ku, into one cell of 2-cell embryos, and examined their effects on subsequent development. For technical reasons, we employed *Lytechinus pictus* in these studies rather than *Arbacia punctulata.* Hence, it was necessary to demonstrate that the fertilized eggs and early embryos of this species would express DNA-PK, and that this enzyme could be activated by the same means as those employed in the *Arbacia.* The first monoclonal antibody against the Ku protein, Ku 162, is an IgG that recognizes a conformational epitope on the Ku heterodimer. It has been used successfully to immunodeplete the DNA-PK activity from *Xenopus* oocytes (Kanungo *et al.*, 1997). When PAS beads are coated with this antibody, the enzyme in cytoplasmic extracts of *L. pictus* 2-cell embryos associates with the beads and is catalytically active in the absence of (ds)DNA (Fig. 1A). This antibody could not be employed in western analysis because it detects the Ku heterodimer, but neither of its subunits. However, a western blot of the proteins eluted from the PAS beads after labeling with [^{32}P]-gamma ATP shows that the interacting catalytic subunit of DNA-PK (p460) is present and can undergo autophosphorylation (Fig. 1B). An immunoblot of the immunoprecipitates made with a monoclonal antibody raised against the human p460 shows that a polypeptide identical to p460 is co-immunoprecipitated by Ku 162 antibody from the cytoplasmic extracts prepared from 2-cell embryos of *L. pictus* (Fig. 1B, lanes 5 and 6).

Based on these results, initial experiments were carried out to determine the effect of the injection of the antibody, Ku 162, or purified DNA-PK, into one cell of a 2-cell embryo. The injection protocol is illustrated in Figure 2A. A

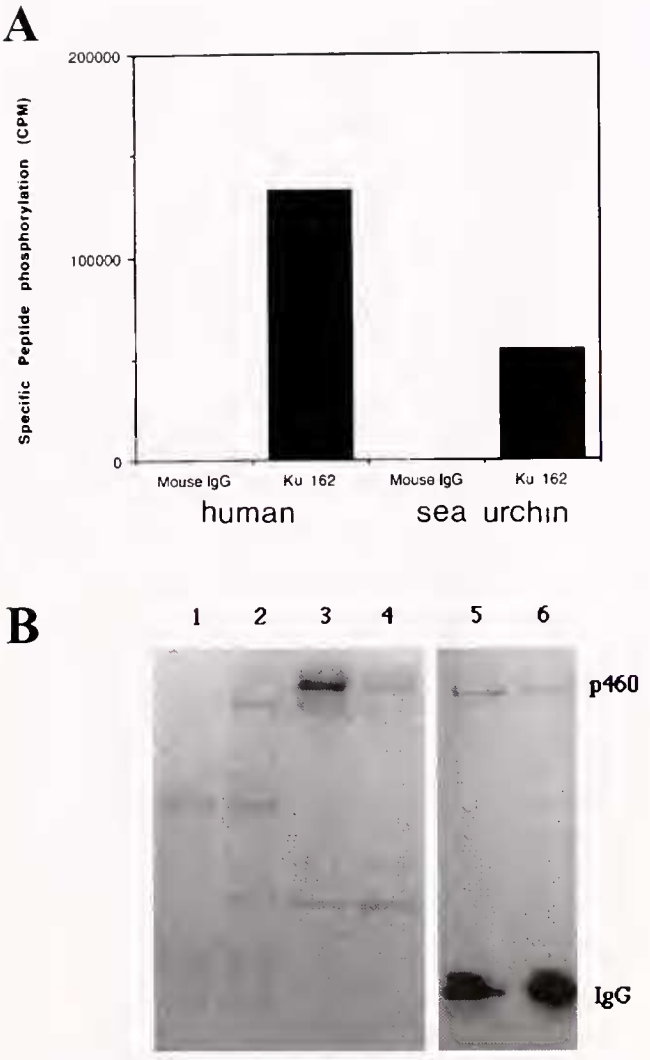

Figure 1. (A) DNA-PK activity of immunoprecipitates of human purified DNA-PK, and of the cytoplasmic extracts of 2-cell embryos of *Lytechinus pictus*. The DNA-PK assay was performed on washed PAS beads coated with the Ku 162 antibody. Reactions for a single experiment were run in duplicate. A representative assay (of five assays) is shown. Note that specific peptide phosphorylation activity is present in immunoprecipitates of cytoplasmic extracts of the sea urchin embryo. The activity from 250 embryos was a little less than half of that obtained from the immunoprecipitates of 25 units of purified human DNA-PK. PAS beads coated with preimmune mouse IgG did not immunoprecipitate any DNA-PK activity. (B) Autoradiograph of proteins recovered from the PAS beads coated with the Ku 162 antibody (lanes 1–4) showing autophosphorylated p460. Preimmune mouse IgG immunoprecipitated no labeled protein from purified human DNA-PK (lane 1) or from cytoplasmic extracts prepared from the two-cell-stage embryos of *L. pictus* (lane 2). The autophosphorylated DNA-PK catalytic subunit (p460) was present in Ku 162 immunoprecipitates of the human enzyme (lane 3), and it was present in Ku 162 immunoprecipitates of cytoplasmic extracts from 2-cell embryos of *L. pictus* (lane 4). Immunoblots of proteins eluted from lanes 3 and 4 are shown in lanes 5 and 6. The p460 polypeptide, eluted from Ku 162 immunoprecipitates of purified human DNA-PK on the immunoblot, was recognized by a monoclonal antibody, mAb 42-26 (Carter *et al.*, 1990) against human p460 (lane 5); a similar protein, from the Ku 162 immunoprecipitates of cytoplasmic extracts of *L. pictus* 2-cell embryos, was also recognized by this antibody (lane 6).

concentration of Ku 162 was chosen (2.5 pg) that completely arrested cell division in about one-half of the injected cells in 2-cell embryos 1 h after fertilization ($n = 17$). At that time, most of the uninjected embryos were at the 16/32 cell stage of development. On the other hand, further progression of the injected cell was completely arrested in one-half of the embryos ($n = 17$) injected with 2.5 pg of Ku 162 (Fig. 2B). Some cells were injected with a standard, but not maximally effective, concentration of purified DNA-PK either in the presence or absence of Ku 162. Injection of purified DNA-PK (the holoenzyme) alone had no effect on the development of the injected cells ($n = 12$, data not shown). However, when the holoenzyme was co-injected with Ku 162, the normal Ku 162-dependent inhibition of cell development was overcome, and 90% ($n = 10$) of the co-injected cells developed normally (Fig. 2C). In other cells, injection of either 0.5 *M* KCl ($n = 13$) or anti-RNA polymerase II antibody (Promega) ($n = 15$) had no effect on the ability of the antibody, Ku 162, to induce developmental arrest.

The further effects of the antibody, Ku 162, on embryonic cell development were analyzed in several ways: serial dilutions of Ku 162 were used to construct a dose-response curve; the effect of the second antibody, N3H10, was determined; and that of preimmune mouse IgG was examined. In addition, the possibility that Ku 162 was inhibiting cell division by causing cell necrosis or apoptosis was evaluated.

As shown in Figure 3A, the injection of standard aliquots of serially diluted Ku 162 antibody solution into one cell of a 2-cell embryo produced two effects on cell development. First, the number of cells that are completely arrested decreased as the amount of injected Ku 162 decreased (Fig. 3A, B, C). Second, at the highest concentration of Ku 162 (5 pg/cell) the development of all injected cells ($n = 21$) was completely arrested at the single-cell stage (Fig. 3A). The injection of 2.5 pg/cell of the Ku 162 antibody completely arrested development of 40% of the cells ($n = 18$) (Fig. 3A, right), and the remaining 60% of injected cells ($n = 17$) displayed variable rates of development (Fig. 3B, left). The injection of 1 pg of Ku 162 had no discernible effect on 50% of cells; *i.e.*, there was no evidence of developmental arrest (Fig. 3C, left). In this case, only 10% of cells showed a complete inhibition of cell division, and 40% displayed variable degrees of retarded development (Fig. 3C, right). To determine if Ku 162 antibody acts by inhibiting cell division, and not by causing cell necrosis or apoptosis, a number of the cells of embryos ($n = 12$) that displayed complete arrest were counter-stained with Hoechst 33342. Evidence of apoptosis was not seen in any of the arrested cells (Fig. 3A, right).

In contrast to the results with the Ku antibody 162, injection of comparable amounts of N3H10, a Ku antibody directed against the 70-kDa subunit of Ku, had no apparent effect on the development of the injected cell of a 2-stage

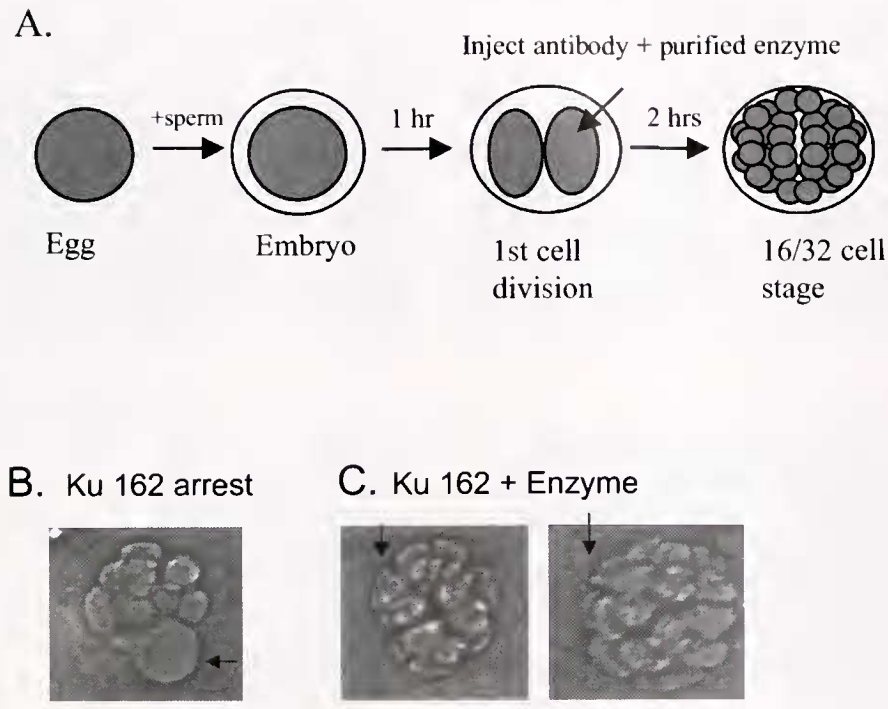

Figure 2. The effect of the antibody, Ku 162, with or without the simultaneous injection of the DNA-PK holoenzyme, on the ability of the injected cell in a 2-cell embryo of *Lytechinus pictus*. Injected cells are identified with an arrow. (A) A diagram of the experimental protocol. (B) The injection of the Ku 162 antibody (2.5 pg/cell) into one cell of a 2-cell embryo caused complete arrest of cell division in the injected cell, but not in the non-injected cell. (C) Two cells, one in each of a 2-cell stage embryo, microinjected simultaneously with Ku 162 antibody (2.5 pg/cell) and purified DNA-PK holoenzyme (4 pg/cell). In neither embryo was arrest or delay of cell division seen in the injected cells, or in the uninjected cells.

embryo (n = 15). It is of interest that one of the subunits (Ku 86) of Ku, but not the other (Ku 70), affected the postfertilization cleavage in sea urchin. This differential function specific to Ku 86 is consistent with the observation that potential functions of DNA-PK, such as X-ray sensitivity, is restored by Ku 86 even when Ku 70 is absent (Smider *et al.*, 1994; Taccioli *et al.*, 1994). An obvious question is whether these two Ku antibodies have different effects on the activity of DNA-PK. On the one hand, we have found that Ku N3H10, when put on PAS beads, is incapable of immunoprecipitating DNA-PK in an active form. What is not yet clear is whether either antibody inhibits DNA-PK *in situ* or in cytoplasmic extracts of sea urchin embryos. Attempts to address this question by using cytoplasmic extracts have proven difficult for several technical reasons, so at present we have no answer.

The present results show that a sufficient concentration of an antibody specific against the Ku heterodimer, Ku 162, when injected into the cytoplasm of one cell of a two-cell sea urchin embryo, completely blocked later cell divisions of the injected cell (Fig. 3). The simultaneous injection of the holoenzyme rescued the injected cells from developmental arrest (Fig. 3C). At intermediate doses, the effects of the Ku 162 antibody were attenuated; *i.e.*, the number of cells completely arrested was reduced, and a number of cells were delayed, but not completely arrested, in their development (Fig. 2B, C). Although the simultaneous injection of the holoenzyme rescued the cells injected with Ku 162 from developmental arrest, injection of the holoenzyme itself had no consistent effect. In these rescue experiments, however, the purified holoenzyme, which is quite labile, appears to be acting as an antigen rather than as an active enzyme. In this light, the lack of effect when the holoenzyme is injected should not be taken as evidence that endogenous active enzyme has no effect on cell cycle progression. Additional experiments will be needed to test this issue in a critical fashion.

An inhibition of mitosis in sand dollar embryos follows the microinjection of antibodies against the calcium transport enzyme of muscle (Silver, 1986). And a similar inhibition occurs in sea urchin embryos after the microinjection of antibodies against kinesin-like proteins (Wright *et al.*, 1993), the mitotic calcium transport system (Hafner and Petzelt, 1987), or a 62-kDa mitotic apparatus protein (Dinsmore and Sloboda, 1989). On the other hand, a controlled translocation of other nuclear enzymes, like DNA polymerase (Loeb *et al.*, 1970), from cytoplasmic stores to the nucleus occurs during sea urchin embryogenesis. Recently,

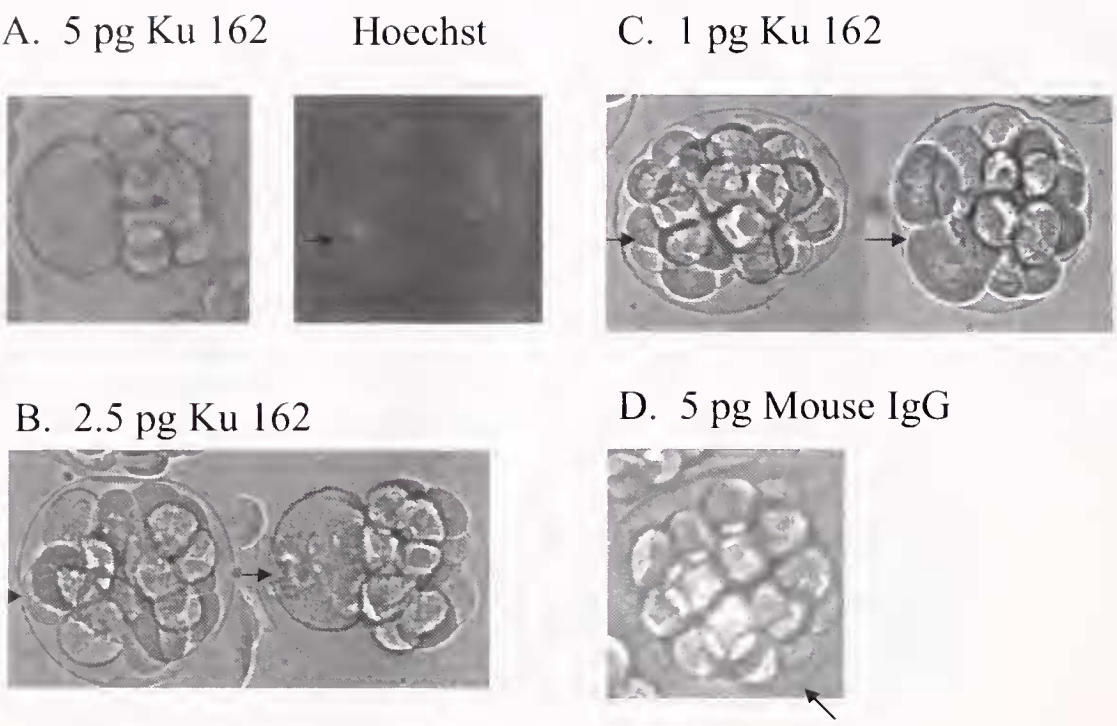

Figure 3. The dose-response effects of the Ku 162 antibody on the arrest of cell division in one cell of a 2-cell stage embryo of *Lytechinus pictus*. (A) When 5 pg/cell of the Ku 162 antibody was microinjected into one cell of a 2-cell embryo, the further development of the injected cells was completely inhibited (left). Shown is an injected cell (arrow) that persists as a single cell, whereas the non-injected cell of the 2-cell stage embryo has undergone further cell divisions. Also shown (right) is an embryo in which Ku 162 had arrested development of the injected cell, and the cell was then labeled with the DNA stain Hoechst 33342. In all arrested cells tested, the DNA could be seen as a single entity (arrow). (B) The injection of 2.5 pg of Ku 162 antibody (see arrow) led to complete arrest in some injected cells (right), whereas the remaining displayed variable rates of cell division. Typical embryos with complete inhibition of cell developmental arrest (right), and partial development arrest (left) are shown. (C) At the lowest concentration of Ku 162, 1 pg/cell, nearly half of the embryos displayed normal rates of cell division (left), 10% displayed complete arrest, and the remainder displayed variable rates of development (right). (D) The effect of preimmune mouse IgG on development. One cell of the 2-cell embryo was injected with 5 pg of preimmune mouse IgG. Of 23 embryos injected with IgG, 23 went on to divide normally. A representative picture of one such cell is shown. The arrow indicates the location of the injected cell.

it has been reported that the catalytic activity of DNA-PK does not require Ku (Yaneva *et al.*, 1997; Hammerstein and Chu, 1998). The simplest hypothesis to account for our result is that the Ku 162 antibody interferes with a temporally controlled transport of DNA-PK from a cytoplasmic store to the nucleus as development proceeds. Alternatively, this antibody may inhibit the activity of the enzyme in either a nuclear or a cytoplasmic location. In any case, our results suggest that Ku plays an important role in the control of cell cycle events during early embryogenesis.

Acknowledgments

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